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MicroRNA-20 induces methylation of hepatitis B virus covalently closed circular DNA in human hepatoma cells

간암세포에서 microRNA-20이 B형간염 바이러스 covalently closed circular DNA의 메틸화에 미치는 영향

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ABSTRACT

Background: Methylation suppresses transcriptional activity of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) in hepatocytes and this may contribute to its low replicative activity during the inactive stage of chronic HBV infection. However, the exact mechanisms of methylation are not known. It has been reported short hairpin RNAs induce methylation of the HBV genome in hepatoma cell lines, and that the microRNA (miR) 17-92 cluster negatively regulates HBV replication in human hepatoma cells. Moreover, miR-20a, a member of the miR 17-92 cluster, has sequence homology with the short hairpin RNA that induces HBV methylation. In this study, I tested the hypothesis that miR-20a functions as an endogenous effector of HBV DNA methylation.

Method: HepAD38 cells, a cell line which permits HBV replication under the control of tetracycline-responsive promoter were used for in vitro HBV replication model. MicroRNA expression plasmids containing precursor sequences of the miR-17-92 cluster were transfected to HepAD38 cells. Methylation of HBV covalently closed circular DNA was assessed by methylation-specific PCR, and HBV relaxed circular DNA was quantified by real-time qPCR and Southern blotting. Quantification of cytoplasmic HBV pregenomic RNA was performed by real-time RT-PCR and dot blot analysis. After actinomycin D treatment, HBV pgRNA was measured to examine RNA stability by real-time RT-PCR. The association of Argonautes (AGOs) with HBV cccDNA was assessed by chromatin immunoprecipitation (ChIP). The presence of nuclear AGO-miR complex was assessed by ribonucleoprotein immunoprecipitation (RNP-IP).

Results: Over-expression of miR-20a suppressed the replicative activity of HBV and increased the degree of methylation of HBV cccDNA in the HepAD38 hepatoma cell line. AGO1 and AGO2, effectors of RNA-induced silencing complex, were found in the nucleus of HepAD38 cells. However, only AGO2 was bound to HBV cccDNA, and intranuclear AGO2 was loaded with miR-20a.

Conclusion: MiR-20a is loaded onto AGO2, translocated into the nucleus, and induces methylation of hepatitis B virus DNA in human hepatoma cells, leading to suppression of HBV replication.

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Key words: hepatitis B virus; methylation; microRNA; Argonaute; covalently closed circular DNA

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LIST OF ABBREVIATIONS

HBV: Hepatitis B virus

cccDNA: covalently closed circular DNA

rcDNA: relaxed circular DNA

miR: microRNA

AGO: argonaute

NTCP: sodium taurocolate cotransporting polypeptide

CHB: chronic hepatitis B

RISC: RNA-induced silencing complex

DNMT: DNA methyltransferase

RdDM: RNA-directed DNA methylation

pgRNA: pregenomic RNA

SSC: standard saline citrate

RT-PCR: reverse transcription-PCR

ChIP: chromatin immunoprecipitation

RNP-IP: ribonucleoprotein immunoprecipitation

TGS: transcriptional gene silencing

HBx: hepatitis B virus X protein

INTRODUCTION

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family (1). The infectious virus particle, called Dane particle, consists of an outer lipid envelope and inner nucleocapsid. The nucleocapsid encloses the viral genome, relaxed circular DNA (rcDNA), and viral polymerase that has reverse transcriptase activity (2). The outer envelope contains glycoproteins which are involved in viral binding and entry of susceptible cells. HBV binds to receptor on the cell surface and enters endocytosis into the cells (3). HBV initially binds nonspecifically to heparan sulfate proteoglycan and specifically binds to the cell surface receptor, sodium taurocolate cotransporting polypeptide (NTCP) (4). Following endocytosis, the virus releases the nucleocapsid into the cytoplasm (5). Because the virus amplifies via RNA, the viral DNA has to be transferred to the nucleus. When rcDNA move into the nucleus, the partially double stranded viral DNA transformed into covalently closed circular DNA (cccDNA) that serves as a template for transcription of viral mRNAs.

Hepatitis B virus (HBV) poses global health burden by infecting more than 250 million people worldwide (6). Although the global prevalence of acute HBV infection is decreasing due to effective immunization, chronic hepatitis B (CHB) and other HBV-associated chronic liver diseases have not decreased during the past decade (7). One of the main challenges in HBV eradication is the persistence of viral minichromosome, also known as cccDNA which can stably remain in the nucleus of the infected hepatocytes even after prolonged effective

antiviral therapy (8, 9). For this reason, HBV cccDNA has emerged as the main target molecule to achieve complete cure from chronic HBV infection (10).

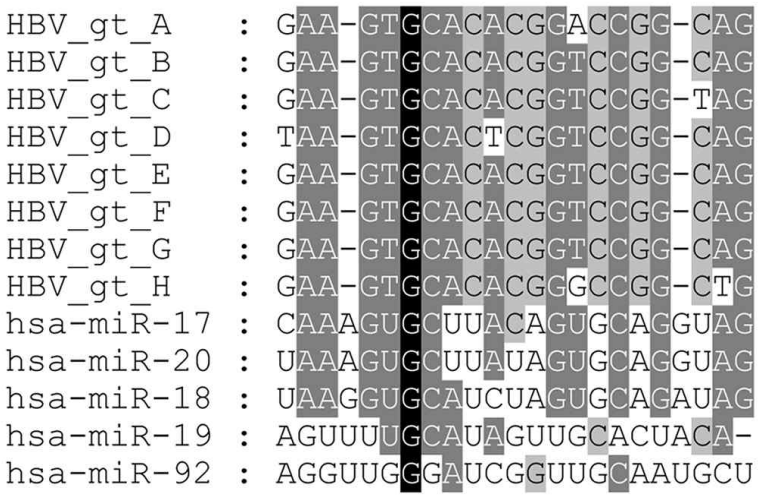
The natural course of CHB has characteristic stages with respect to viral loads and hepatic inflammation: the initial immune tolerant phase with high viral loads and normal hepatic inflammation markers is followed by an immune clearance phase with flare of hepatic inflammation and decrease in viral DNA levels, and finally the immune control phase with low or undetectable circulating viral loads ensues (11, 12). Progressive decrease in the serum HBV viral loads may be attributed to the decrease in the amount of nuclear HBV cccDNA. However, HBV cccDNA persists throughout the natural course of CHB even in patients with complete virologic and serologic clearance, suggesting that the viral productivity of HBV cccDNA may decrease over time (9, 12). Indeed, measurement of intrahepatic HBV RNA levels has demonstrated that the transcriptional activity of cccDNA decreases in the immune control phase of CHB (13, 14). The transcriptional activity of HBV cccDNA is modulated by various non-specific and liver-specific transcription factors (13). In addition, epigenetic modifications of HBV cccDNA, such as histone modifications and methylation of HBV cccDNA may also affect cccDNA transcription (15-17). Previously, studies by our group as well as other researchers have reported that methylation of HBV cccDNA contributes to transcriptional control of HBV cccDNA in CHB (18-20). However, the exact mechanism(s) by which the HBV cccDNA is methylated has not been elucidated yet.

MicroRNA (miRNA) is a small non-coding RNA found in plants, animals, and some viruses (21-23). MiRNAs regulate mammalian gene expression at the

post-transcriptional level by directing RNA-induced silencing complex (RISC) to complementary mRNA targets and subsequently inducing translational repression and/or mRNA destabilization (24, 25). Potential target sites of these miRNAs were found in the HBV genome using a bioinformatics program (40). MiRNAs may also regulate gene expression at the transcriptional level indirectly by modifying the expression of DNA methyltransferases (DNMTs) (26, 27). In plants, miRNAs directly induce transcriptional silencing through DNA methylation by recruiting Argonaute (AGO)-containing effector complexes (28-32) to the target DNA. It is not yet known whether miRNAs can directly induce transcriptional silencing in mammalian cells. It is plausible that similar miRNA machineries may also work, analogous to RNA-directed DNA methylation (RdDM) by small interfering RNA (33) or Piwi-interacting small RNA (34, 35) in mammalian cells.

Previous studies have shown that short hairpin RNA induces methylation of HBV cccDNA in hepatoma cells (36). Interestingly, the target sequence of the short hairpin RNA overlaps with the potential target site of hsa-miR-20a, a member of the oncogenic miR-17-92 cluster (37-39). Previous report have also shown that the miR-17-92 cluster negatively regulates of HBV replication in human hepatoma cells (40), and the HBV X sequence shows conserved homology to miR-20a across HBV genotypes (Fig. 1).

Figure 1. Alignment of hepatitis B virus and miR-17-92 cluster miRNAs



Hepatitis B sequences indicate nt1567-1587 on the X gene of each genotype.

Considering the structural homology between short hairpin RNAs and miRNA precursors, I hypothesized that miR-17-92 also induces methylation of HBV cccDNA. To test this hypothesis, I transduced miR-17-92 precursors into human hepatoma cells and studied the methylation profiles of the HBV genome.

MATERIALS AND METHODS

In vitro HBV replication model and isolation of HBV cccDNA

I used HepAD38 cells that support HBV replication under the control of a tetracycline-inducible promoter (a generous gift from Professor C. Seeger, Fox Chase Cancer Center, PA, USA) (41). HBV replication was induced by omitting tetracycline from the culture medium for 5-7 days. HBV cccDNA was purified as previously reported with minor modifications (36). Briefly, HepAD38 cells were lysed by the addition of 0.4 ml of cell lysis buffer (50mM Tris-HCl [pH 8.0], 1mM EDTA, 0.2% [v/v] Nonidet P-40 and 0.15M NaCl). The lysate was centrifuged at 16000 g at 4℃ for 2min, and the supernatant (cytoplasmic fraction) was saved for the extraction of HBV rcDNA (see below). The nuclei pellet was treated with the same volume of nuclear lysis buffer (6% SDS, 0.1N NaOH) at 37℃ for 15 min, phenol-extracted and ethanol-precipitated. Contaminating genomic DNA was removed by treatment with Plasmid-Safe DNase (Epicentre Biotechnologies, Madison, WN), and the absence of contaminating genomic DNA was confirmed by the negative PCR results obtained using a primer pair for β-globin.

Over-expression of miR-17-92

Plasmids expressing human miRNA precursor were purchased from OriGene Technologies (Rockville, MD, USA): pCMV-MIR control, pCMV-MIR17-5p (SC400201), pCMV-MIR18a (SC400218), pCMV-MIR19a (SC400253), pCMV-MIR20a (SC400269) and pCMV-MIR92a (SC400682). Plasmids were transfected into HepAD38 cells using Lipofectamine 2000 (Thermo Fisher scientific Korea). After transfection, the cells were incubated at 37℃ in 5% CO2 for 5 days before carrying out assays for HBV replication and methylation as described below.

Quantification of cytoplasmic HBV DNA by real-time PCR and Southern blotting

Core particle-associated cytoplasmic HBV relaxed circular DNA (rcDNA) was extracted as reported with minor modifications (42). Briefly, HepAD38 cells grown on a 60-mm culture dish were lysed and nuclei were pelleted as described above. Cytoplasmic fraction was treated with 1/4 volume of 35% PEG8000 in 1.75M NaCl, incubated on ice for 30 min and centrifuged at 16000 g for 10min. The pelleted HBV core particles were dissolved in proteinase K buffer (10mM Tris-HCl [pH 8.0], 100mM NaCl, 1mM EDTA, 0.5% SDS, 200 ㎍/㎖ proteinase K) and incubated at 45℃ for one hour. The core-associated rcDNA was recovered by phenol extraction and ethanol precipitation.

Real-time PCR was performed as previously reported to quantify HBV relaxed circular DNA (rcDNA) (43). PCR amplification was performed with SYBR Green and the following primer pair at final concentration of 200 nM: forward, GAGTGTGGATTCGCACTCC and reverse, GAGGCGAGGGAGTTCTTCT. Thermal Cycler Dice Real Time System (Takara BIO) was used according to the manufacturer's instructions. PCR cycling program consisted of an initial denaturing step at 95℃ for 15min, followed by 45 amplification cycles at 95℃ for 10s, 60℃ for 20s, 72℃ for 30s. Statistical significance for the relative quantification was calculated by REST software (Qiagen).

Southern blotting for HBV rcDNA was performed as previously reported (21). Briefly, 15 μg of HBV rcDNA was electrophoresed in 1% agarose/Tris-Borate EDTA gel, followed by partial depurination with HCl (0.25M) for 15 min and denaturation in NaOH (0.5M) for 15 min. DNA was transferred in 20X standard saline citrate (SSC) buffer to a Hybond-N+ membrane (Roche, Mannheim, Germany) by capillary blotting. The membrane was UV-crosslinked and hybridized with digoxygenin-tagged HBV RNA probes in Dig Easy Hybridization Buffer (Roche, Mannheim, Germany) overnight at 60℃. Following hybridization, the membrane was washed twice for 5min at room temperature in 2X SSC, 0.1% SDS and twice for 15min at 60℃ in 0.1X SSC, 0.1% SDS. The membrane was blocked with blocking reagent (Roche, Mannheim, Germany) for 30min at room temperature and was incubated for 30min in blocking buffer containing 187.5 mU/ml (1:4,000 v/v) anti-digoxigenin-AP (Roche, Mannheim, Germany). The membrane was treated with Immun-Star AP Substrate (Bio-Rad Laboratories, CA, USA) and was exposed to X-ray film for 1hr. In order to

estimate the viral productivity of cccDNA, the loading amounts of HBV DNA were normalized by the copy numbers of HBV cccDNA determined by real-time PCR with ΔΔCq methods as previously reported (44, 45).

Quantification of cytoplasmic HBV pregenomic RNA (pgRNA) by real time RT-PCR and dot blot analysis

Total RNA was extracted from the cytoplasmic fraction of HepAD38cells using AccuZol reagent (Bioneer, South Korea) according to the manufacturer's instructions and treated with DNase. Reverse transcription was performed by using 10 μg of total RNA and random hexamers with MMLV (iNtRON Biotechnology, Seongnam, South Korea) as recommended. Real-time reverse transcription-PCR (RT-PCR) was then performed with the same primers for rcDNA amplification, with glyceraldehyde-3-phosphate dehydrogenase used as a normalization control: forward, GCACCGTCAAGGCTGAGAAC and reverse, ATGGTGGTGAAGACGCCAGT.

Dot blot assay was performed in the similar way as Southern blotting. Briefly, 2 μg of cytoplasmic RNA was denatured by NaOH and fixed on the Hybond-N+ membrane by microwave heating for 2min. Hybridization and signal detection with digoxygenin-tagged HBV RNA probe was performed as decribed for Southern blotting.

RNA stability assay

Actinomycin D (5 μg/ml) was added into the culture medium of HepAD38 cells to block transcription at 24 hr after transfection of miRNA mimics (125 nmol). Total RNA was extracted at 1, 3 and 6 hr after actinomycin D treatment and HBV pgRNA was measured by real-time RT-PCR as described above.

Assessment of HBV cccDNA methylation by methylation specific PCR and bisulfite sequencing

HBV cccDNA was isolated as described above and subjected to bisulfite modification using Imprint DNA Modification Kit according to the manufacturer's instructions (Sigma #MOD50). The primers for methylation-specific PCR were designed on the universal CpG island II sequence of HBV (46) by using Methyl Primer Express (v1.0, Applied Biosystems): unmethylation-F, 5′ GTGGGATGTTTTTTGTTTAT 3′; unmethylation-R, 5′ ACAAAATACACACAATCCCAA 3′; methylation-F, 5′ GCGGGACGTTTTTTGTTTAC 3′; methylation-R, 5′ ACGAAATACACACGATCCGA 3′ at final concentration of 200nM. The PCR cycling program consisted of an initial denaturation at 95℃ for 15min, followed by 45 amplification cycles at 95℃ for 10s, 55℃ for 20s and 72℃ for 30s. Bisulfite sequencing was also performed by PCR cloning of bisulfite modified HBV cccDNA CpG island II as previously reported (36).

Chromatin immunoprecipitation and ribonucleoprotein immunoprecipitation

The association of Argonautes (AGOs) with HBV cccDNA was assessed by chromatin immunoprecipitation (ChIP) (47, 48) with modifications. Briefly, plasmids expressing FLAG-AGO1 and FLAG-AGO2 protein (Addgene plasmid # 21533 and # 21538, respectively; gifts from Edward Chan) (49) were transfected to HepAD38 cells. On day 5, cells were cross-linked using 1.42% formaldehyde for 15min at room temperature. Nuclei were isolated and sonicated as described (47). Chromatin samples were treated with anti-FLAG M2 antibody (Sigma F1804) and pre-incubated with Dynabeads Protein G (Thermo Fisher scientific) as recommended. DNA was eluted from the washed beads by Chelex100 (Bio-Rad, cat. no. 142-1253) boiling method (48). HBV cccDNA was detected using specific primers (50) after treatment with Plasmid-safe Dnase (Epicentre E3101K, Madison, WI).

The presence of nuclear AGO-miR complex was assessed by ribonucleoprotein immunoprecipitation (RNP-IP) as reported (51) with modifications. Briefly, RNaseOut (final 100 U/ml, Thermo Fisher scientific 10777019) and dithiothreitol (final 1mM) were added to the nuclear lysis samples in the ChIP procedure above (47). Nuclear RNA was released from cross-linked proteins by digestion with proteinase K for 1 hr at 55℃, followed by Trizol extraction. Stem-loop RT-PCR was performed to detect miR-20a with primers designed as reported (52): stem-loop RT primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCT-3'

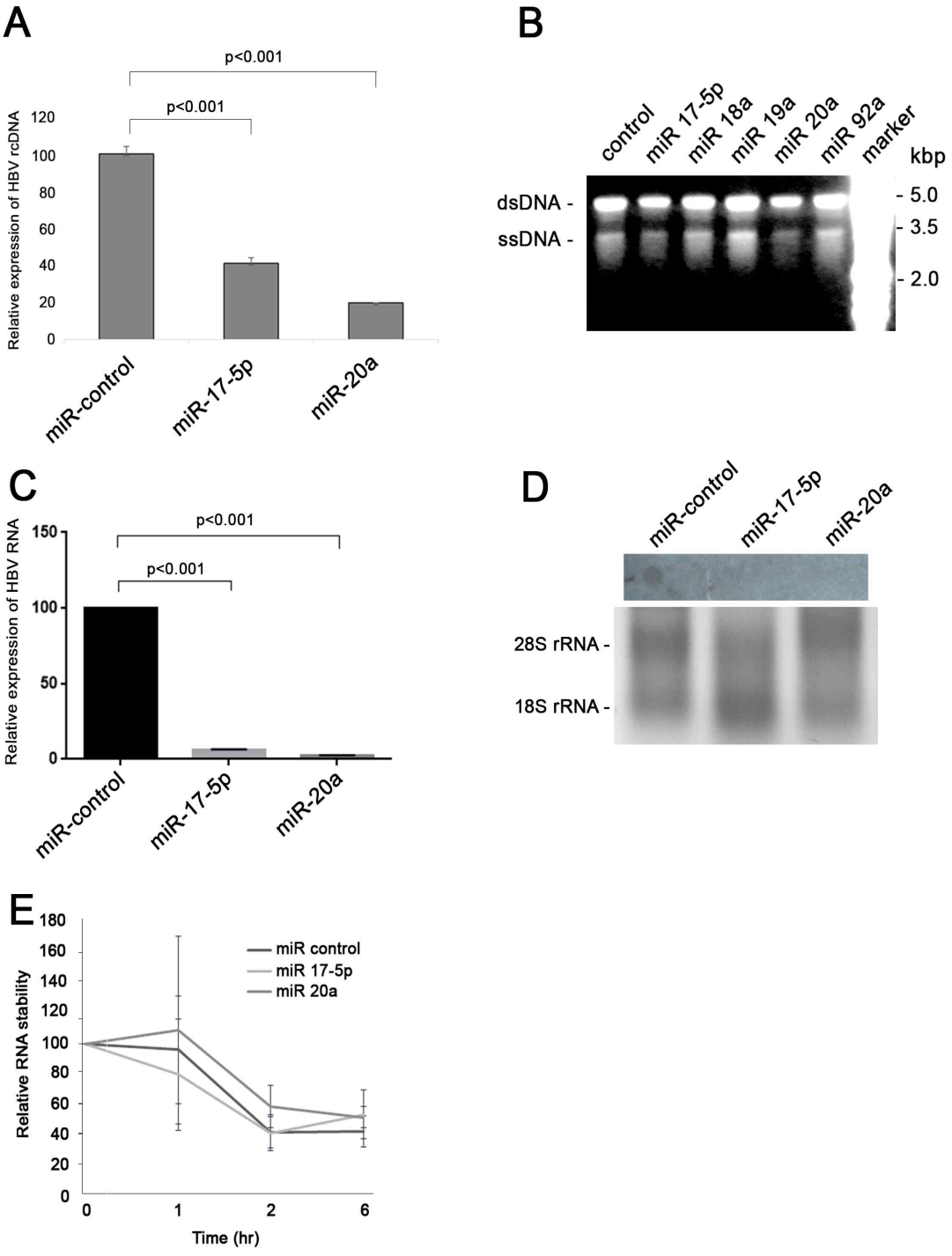
; forward-miR-20a primer, ACACTCCAGCTGGGTAAAGTGCTTAT; reverse primer, CCAGTGCAGGGTCCGAGGTA.

RESULTS

miR-20a suppresses replication of HBV cccDNA at the transcriptional level in HepAD38 cells

Previous study has shown that miR-17-92 cluster negatively regulates HBV replication in human hepatoma cells (40). Real-time PCR and Southern blotting re-confirmed that miR-17-5p and miR-20a reduced the HBV DNA level in HepAD38 cells (Fig. 2A and 2B). The reduction was associated with suppressed HBV RNA level (Fig. 2C and 2D). RNA stability assay showed that degradation of HBV RNA was not accelerated by miR-17-5p and miR-20a (Fig. 2E). The fact that miR-17-5p and miR-20a did not affect the half-life of the HBV RNA indicates that the miRs act on the transcription of HBV RNA.

Figure 2. Over-expression of miR-17-5p and miR-20a suppresses HBV replication in HepAD38 cells.



(A) Cytoplasmic HBV relaxed circular DNA (rcDNA) was measured by real-time PCR in HepAD38 cells transfected with miR-17-5p and miR-20a precursor plasmids. The error bars indicate standard deviation.

(B) Southern blotting analysis of cytoplasmic HBV rcDNA was performed after transfection of miR-17-92 precursor plasmids. The loading amounts were adjusted according to the HBV cccDNA copy numbers measured by real-time PCR. Therefore, the relative quantity of HBV DNA indicates viral productivity per unit cccDNA. Compared to control, miR-17-5p and miR-20a suppressed HBV replication by 37% and 43%, respectively, as evaluated by densitometric measurements. The image is representative of three independent experiments.

(C) Cytoplasmic HBV RNA was measured by real-time PCR in HepAD38 cells transfected with miR-17-5p and miR-20a precursor plasmids. The error bars indicate standard deviation.

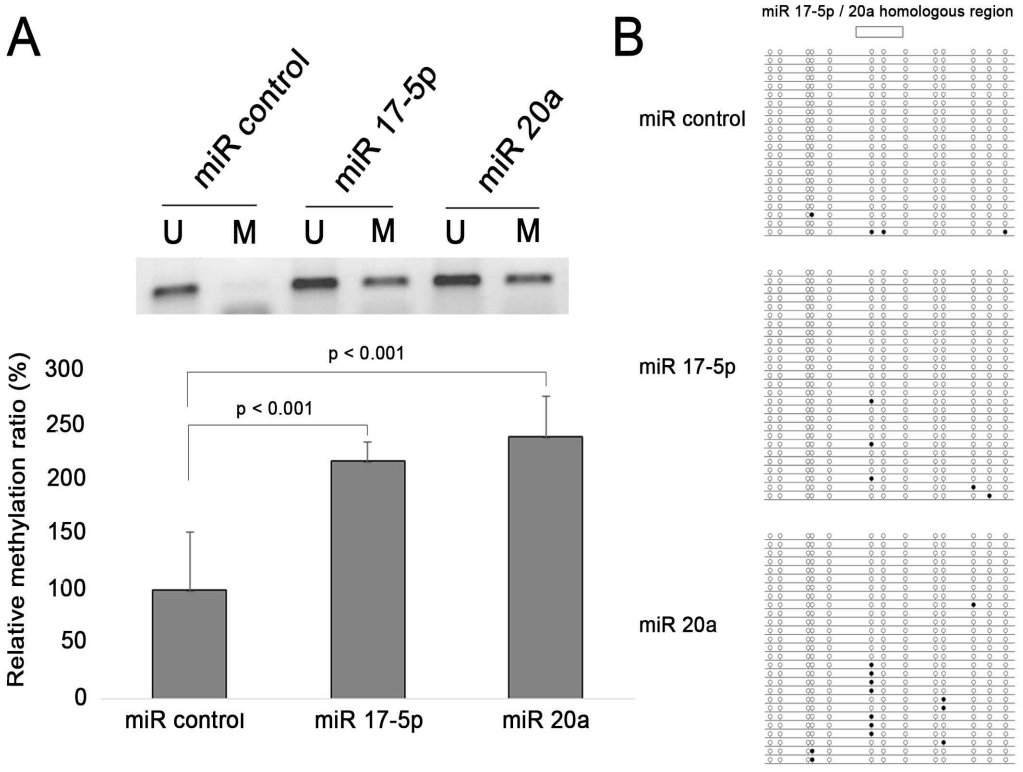
(D) Dot blot assay of HBV RNA confirmed suppression of HBV RNA by miR-17-5p and miR-20a.

(E) Analysis of HBV RNA stability in HepAD38 cells transfected with miR-17-5p and miR-20a precursor plasmids. Actinomycin D was added to culture medium and RNA was harvested at given time points. Error bars indicate standard deviation.

miR-20a induces methylation of HBV cccDNA in HepAD38 cells

Next, I sought to determine whether miR-17-5p and miR-20a induced methylation of HBV cccDNA because these two members of the miR-17-92 cluster miRNAs suppressed replication of HBV at the transcriptional level. Methylation-specific PCR showed that over-expression of miR-17-5p and miR-20a induced a significant increase in the degree of methylation in HBV cccDNA (Fig. 3A). Bisulfite sequencing revealed that the frequency of methylation induced by miR-17-5p was not significantly different from control, whereas miR-20a induced significantly higher level of methylation in the putative target site of HBV cccDNA (Fig. 3B).

Figure 3. miR-20a induces methylation of HBV cccDNA.



(A) Methylation-specific PCR assay. HepAD38 cells were transfected with miRNA precursor plasmids and cccDNA was isolated at day 7 for bisulfite modification. Bisulfite-modified HBV cccDNA was amplified with methylation-specific PCR primer sets. (upper panel; U, unmethylated primer set; M, methylated primer set). The relative amount of methylated to unmethylated cccDNA was assessed by real time PCR (lower panel). The results are representative of three independent experiments; error bars indicate standard deviation.

(B) Bisulfite sequencing analysis of HBV cccDNA. CpG island II was amplified from bisulfite-modified HBV cccDNA, TA-cloned and sequenced. Methylated and unmethylated CpG dinucleotides were marked by open and filled circles, respectively. Compared to control transfection, miR-17-5p showed a similar level of methylation ($p = 0.348$), whereas miR-20a showed a significantly higher level of methylation ($p = 0.001$).

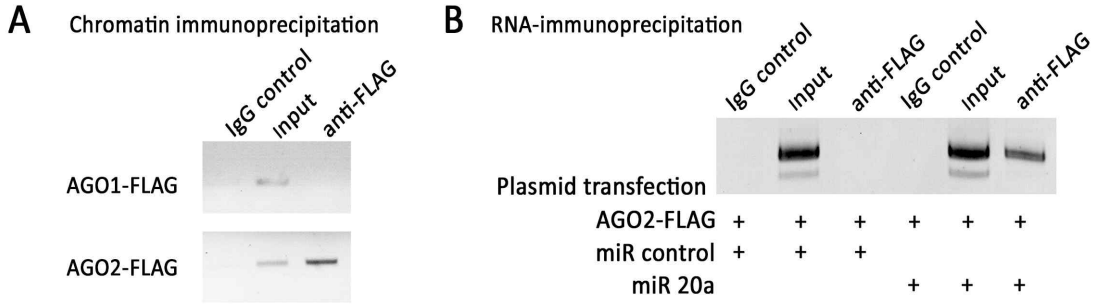
AGO2 is translocated to the nucleus of HepAD38 cells and bound to HBV cccDNA

In order to elucidate the mechanisms of cccDNA methylation by miR-20a, I sought to determine whether AGOs, the main effectors of RNA-induced silencing complex (RISC), are directed to nuclear HBV cccDNA. ChIP assay showed that both AGO1 and AGO2 were present in the nucleus (Fig. 4A, input lanes). However, AGO2 but not AGO1 was associated with HBV cccDNA (Fig. 4A, anti-FLAG lanes).

Nuclear AGO2 associates with miR-20a

Since AGO2 was associated with nuclear HBV cccDNA, RNP-IP was performed to determine whether miR-20a interacts with intranuclear AGO2. I confirmed that over-expression of miR-20a induced the appearance of miR-20a-loaded AGO2 in the nuclei of HepAD38 cells (Fig. 4B).

Figre 4. Nuclear AGO2 is associated with HBV cccDNA and miR-20a.



(A) ChiP analysis was performed using HepAD38 cells transfected with AGO1-FlAG (upper gel) or AGO2-FLAG plasmid (lower gel). Immunoprecipitated DNAs were amplified with HBV cccDNA-specific primers. AGO1 did not bind HBV cccDNA (anti-FLAG lanes, upper gel), whereas AGO2 was associated with HBV cccDNA (anti-FLAG lanes, lower gel). The results represent three independent experiments.

(B) RNP-immunoprecipitation analysis was performed using HepAD38 cells transfected with AGO2-FLAG plasmid, along with miR-expressing plasmid. RNA was extracted from anti-FLAG immunoprecipitates and amplified for miR-20a. The results represent two independent experiments.

Discussion

Although methylation has been identified both *in vitro* (18, 36) and *in vivo* (18-20) as a mechanism of transcriptional regulation of HBV replication, it is not known how HBV cccDNA is methylated. Previous study has also shown that miR-20a interacts directly with HBV X / polymerase gene sequence (40). The potential target sequence was also shared by short hairpin RNA which can induce methylation of HBV cccDNA (36). These findings prompted us to evaluate the epigenetic effect of miR-20a in this study. Indeed, I found that human miR-20a modulate *de novo* methylation of nuclear HBV DNA in a hepatoma cell line. To the best of my knowledge, this is the first report on the methylation of HBV genome by over-expression of microRNA.

RNA-directed transcriptional gene silencing (TGS) has been reported in mammalian cells by independent researchers (33, 58-60), but the underlying mechanisms have not yet been fully understood. Human miRNA is loaded onto RNA-induced silencing complex (RISC) which includes Dicer, AGOs, TRBP and GW182 (53), and miR-loaded RISC acts as an effector of slicer-dependent and slicer-independent post-transcriptional silencing (54). In addition, it has been reported that AGO1 and AGO2 are transported to nuclear compartments of human cells (55). Presence of AGO2 and RNA interference factors in human nuclei suggests their participation in TGS (56, 57, 61). Indeed, nuclear translocation of miR-loaded AGO2 is associated with gene silencing (62), and

AGO2 and miRNAs are recruited to the promoter region leading to TGS (60, 63). In this work, I showed that miR-20a-loaded AGO2 is directed into the nucleus (Fig. 4B), which is in line with the previously suggested hypothesis that TGS can be brought about by miR-loaded AGO2.

My data also demonstrated that AGO2 can bind to the nuclear HBV genome (cccDNA) (Fig. 4A). This finding, along with the translocation of miR-20a-loaded AGO2 into the nucleus, suggests that miR-20a directs AGO/RISC to the homologous target of HBV DNA. I speculate that miR-guided AGO/RISC may work as an effector of *de novo* methylation in mammalian cells, in a way analogous to RdDM in plants (66). Hepatitis B virus X protein (HBx) upregulates DNA methyltransferase 3 (DNMT3) (65), which is the principal enzyme for mammalian *de novo* DNA methylation (66), and directs DNMT3 to target DNA (67). Based on this, I propose that HBV cccDNA first binds to the miR-guided AGO2-RISC, which in turn recruits HBX-driven DNMT3 to AGO2-bound cccDNA. However, further validation is required to prove this hypothesis by studying the interaction between miR-20a-loaded RISC, DNMT3a HBx and HBV cccDNA.

Real-time RT PCR and hybridization assays confirmed that miR-17-5p and miR-20a suppress HBV RNA but do not affect RNA stability. These findings suggest that miR-induced methylation suppresses transcription of HBV cccDNA. Methylation has been shown to suppress transcriptional activity of HBV

cccDNA by *in vitro* nuclear run-off assay (18). Bisulfite sequencing showed that miR-17-5p did not induce statistically significant methylation. Since miR-17-92 cluster miRs have been identified in the mammalian nuclei (68-71), and the mature sequences of miR-17-5p and miR-20a differ by only two ribonucleotides (Fig. 1), miR-17-5p may also induce methylation that may reduce the transcriptional activity of HBV cccDNA, as indicated by suppressed viral productivity observed by Southern hybridization and increased methylation observed by methylation-specific PCR.

Although it is known that methylations of HBV cccDNA contributes to transcriptional suppression of HBV replication (18-20), and yet the exact mechanism(s) of methylation has not been elucidated yet. I believe that my result may shed light on the role of miRNA as an innate epigenetic modulator in chronic HBV infection. Moreover, my findings may help in understanding the endogenous mechanisms of TGS in mammalian cell. As the persistence of HBV cccDNA is the major obstacle in curing the chronic HBV infection (9, 72), these miRNA-induced epigenetic modifications may have therapeutic potential in the development of novel therapy for chronic hepatitis B. Since small RNAs has been approved as a new class of therapeutics, e.g., Patisiran for hereditary transthyretin amyloidosis (73), small RNA-based HBV regulation may be technically feasible for the long-term suppression of HBV.

However, there are still several issues that need to be addressed: 1) the

recruitment of DNMT by the miR-20a/AGO2 complex to HBV cccDNA needs to be confirmed; 2) the presence of miR-induced methylation machinery needs to be ascertained in non-neoplastic hepatocytes infected with HBV; 3) strategies to circumvent the risk for hepatocellular carcinoma should be developed, considering the oncogenic potential of miR-17-92 (39).

In conclusion, I infer that in human hepatoma cells, miR-20a is loaded onto AGO2 and translocated into the nucleus, where it induces methylation of hepatitis B virus DNA, leading to suppression of HBV replication.

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국 문 초 록

배 경: 메틸화는 간세포에서 B형간염 바이러스 (HBV) covalently closed circular DNA (cccDNA)의 전사 활성을 억제하며 이는 만성 HBV 감염의 비활성 단계 동안 낮은 복제 활성에 기여할 수 있다. 그러나 메틸화의 정확한 메커니즘은 아직 알려져 있지 않다. 우리는 사전 연구에서 short hairpin RNA가 간암 세포주에서 HBV 게놈의 메틸화를 유도한다는 것을 보여주었다. 우리는 또한 microRNA (miR) 17-92 cluster가 인간 간암 세포에서 HBV 복제를 억제한다는 것을 발견했다. 또한, miR-17-92의 구성원인 miR-20a는 HBV 메틸화를 유도하는 short hairpin RNA와 서열 상동성을 갖는다. 이 연구에서, 우리는 miR-20a가 HBV DNA 메틸화의 내인성 효과기로서 기능한다는 가설을 증명하였다. 그 결과, miR-20a의 과발현이 HBV의 복제 활성을 억제하고 HepAD38 간암 세포주에서 HBV cccDNA의 메틸화 정도를 증가시키는 것으로 나타났다. HepAD38 세포의 핵에서는 RNA 유도성 침묵 복합체의 효과기인 AGO1과 AGO2가 발견되었다. 그러나, AGO2만이 HBV cccDNA에 결합되었고, 핵 내 AGO2에는 miR-20a가 결합되었다.

실험방법: 테트라사이클린 반응성 프로모터의 조절하에 HBV를 복제하는 세포주인 HepAD38 세포를 HBV 복제 모델에 사용하였다. MiR-17-92 cluster의 전구체 서열을 포함하는 microRNA 발현 플라스미드를 HepAD38 세포에 형질 감염시켰다. 메틸화 특이적 PCR에 의해 HBV의 covalently closed circular DNA의 메틸화를 측정하였고, real-time qPCR 및 southern blotting으로 HBV relaxed circlar DNA를 정량하였다. 세포질 HBV pregenomic RNA의 정량은 real-time RT-PCR 및 dot blot 분석에 의해 수행되었다. Actinomycin D 처리 후 real-tie RT-PCR을 이용하여 HBV pgRNA를 측정하여 RNA의 안정성을 조사하였다. Argonautes (AGOs)와 HBV cccDNA의 연관성을 염색질 면역 침전 (ChIP)으로 평가하였다. 핵 AGO-miR

복합체의 존재는 ribonucleoprotein immunoprecipitation (RNP-IP)에 의해 평가되었다.

결　　과: MiR-20a의 과발현은 HBV의 복제 활성을 억제하고 HepAD38 간암 세포주에서 HBV cccDNA의 메틸화 정도를 증가시켰다. HepAD38 세포의 핵에서는 RNA 유도성 침묵 복합체의 효과기인 AGO1과 AGO2가 발견되었다. 그러나, AGO2만이 HBV cccDNA에 결합되었고, 핵 내 AGO2에는 miR-20a가 로딩되었다.

결론 : MiR-20a는 AGO2로 옮겨져 핵으로 옮겨지고 인간 간암 세포에서 B형간염 바이러스 DNA의 메틸화를 유도하여 HBV 복제를 억제합니다.

결　　론: MiR-20a는 AGO2로 옮겨져 핵으로 옮겨지고 인간 간암 세포에서 B형간염 바이러스 DNA의 메틸화를 유도하여 HBV 복제를 억제한다.

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주요어: B형간염 바이러스; 메틸화; microRNA; Argonaute; covalently closed circular DNA

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